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# ENCYSTATION: THE MOST PREVALENT AND UNDERINVESTIGATED DIFFERENTIATION PATHWAY OF EUKARYOTES.

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## Abbreviations:

MRSA: Methicillin-Resistant *Staphylococcus aureus*; LCA: last common ancestor; HSP: heat shock protein; CP: cysteine protease; RNAi: RNA interference; PHMB: polyhexamethylene biguanide; EST: expressed sequence tag; PRMT5: protein arginine methyltransferase 5; BAR:  $\beta$ -adrenergic receptor; PKC: protein kinase C; cAR: cAMP receptor; PKA: protein kinase A; PkaC: PKA catalytic subunit; PkaR: PKA regulatory subunit; ACA: adenylate cyclase A; ACG: adenylate cyclase G; ACR: adenylate cyclase R; PDE: cAMP phosphodiesterase; SHKP: sensor histidine kinase/phosphatase; SDF2: spore differentiation factor 2; cNMP: cyclic nucleotide; GPCR: G-protein coupled receptor; SH2: src homology domain 2.

## Summary

Not long ago protists were considered one of four eukaryote kingdoms, but recent gene-based phylogenies show that they contribute to all nine eukaryote subdomains. The former kingdoms of animals, plants and fungi are now relegated to lower ranks within subdomains. Most unicellular protists respond to adverse conditions by differentiating into dormant walled cysts. As cysts, they survive long periods of starvation, drought and other environmental threats, only to re-emerge when conditions improve. For protists pathogens, the resilience of their cysts can prevent successful treatment or eradication of the disease. In this context, effort has been directed towards understanding the molecular mechanisms that control encystation. We here firstly summarize the prevalence of encystation across protists and next focus on Amoebozoa, where most of the health related issues occur. We review current data on processes and genes involved in encystation of the obligate parasite *Entamoeba histolytica* and the opportunistic pathogen *Acanthamoeba*. We show how the cAMP mediated signalling pathway that controls spore and stalk cell encapsulation in *Dictyostelium* fruiting bodies could be retraced to a stress-induced pathway controlling encystation in solitary Amoebozoa. We highlight the conservation and prevalence of cAMP signalling genes in Amoebozoan genomes and the suprisingly large and varied repertoire of proteins for sensing and processing environmental signals in individual species.

## Introduction

Environmental change, be it weather-related, seasonal or through disappearance of ephemeral habitats, is constantly encountered by all living organisms, except perhaps parasites and those in stable marine environments. A common response of many protists to environmental stress is differentiation of actively feeding trophozoites into dormant walled cysts. Cysts can be asexual, resulting from the encapsulation of a single cell, or sexual resulting from the encapsulation of a zygote, formed by fusion of two cells of opposite mating types. In the latter case meiotic and mitotic divisions usually occur before the cyst germinates. When a cyst is carried aloft on a stalk or part of a larger multicellular structure, it is more commonly called a spore. However, other descriptions, such as hypnospore, resting spore, zygospore, hypnozygote, oospore are also in use to describe the asexual or zygotic cysts of different groups of unicellular protists. Frequently, the function of cysts or spores is not only the survival, but also the dispersal of the organism to new feeding grounds.

Encystment of protists is of immense ecological importance, allowing phytoplankton to survive long winter darkness and all protists at high latitude and altitude the freezing of their habitats, even for thousands of years [1]. Encystment is also relevant for human health because encysted pathogens, such as *Acanthamoeba castellanii* are resistant to antibiotics, antiseptics and high levels of UV and gamma radiation [2, 3]. Cysts also resist immune attack, because they do not attract neutrophils or macrophages to the site of infection [4]. Additionally, predatory Amoebozoa that feed on bacteria, are often exploited as hosts by bacterial pathogens, such as *Legionella pneumonila*, *Vibrio cholerae*, *Mycobacterium leprae* or Methicillin-Resistant *Staphylococcus aureus* (MRSA) that enter by normal phagocytosis, but manage to avoid digestion by lysosomes. After encystation, the cysts act as vectors for airborne dispersal and survival of the pathogens in man-made ducting and water reservoirs [5-9].

Despite its ecological and medical relevance, but particularly its importance as the major and often single differentiation pathway of protists, only limited information is available about the molecular mechanisms controlling encystation. With this review we summarize the prevalence of encystment as a strategy for survival and dispersal across protists and discuss

existing information on the mechanisms controlling encystation in Amoebozoa, where the health implications of encystation are most severe.

### Encystation occurs in all eukaryote domains

In the earlier morphology-based five kingdom classification of living organisms, protists made up one of the kingdoms, in addition to the kingdoms of plants, animals, fungi and prokaryotes [10]. This subdivision was completely overturned by gene-based classification, which continues to be refined by incorporation of multiple genes or even entire genomes into the inference of family relationships between organisms [11, 12]. Modern systematics now highlights that the genetic diversity of prokaryotes greatly exceeds that of eukaryotes and within eukaryotes, the former kingdom of protists is more diverse than plants, animals and fungi together [11]. The mostly single-celled protists are not one of several kingdoms, but participate in all 3 main and 9 subdomains of eukaryotes (figure 1). Animals, plants and fungi emerged within 3 subdomains, representing only a fraction of their genetic diversity. Organisms distributed over most subdomains display differentiation into one or more dormant encapsulated cell types (summarized in figure 1).

*Jakobida*. In this subdomain, cysts were observed in *Reclinomonas americana*, but not in *Jakoba libera* [13].

*Excavata (Discicristata)*. This subdomain harbours the anaerobic parasite *Giardia lamblia*, which feeds as a flagellated trophozoite in the gut and encyst when excreted into the environment [14]. The free-living amoeboflagellate *Naegleria* encysts in response to starvation stress, while *Acrasis* amoebas either encapsulate individually to form cysts when starved, or aggregate to form fruiting bodies with spores. Cysts are absent from the *Trypanosoma* and *Leishmania* parasites, but the related free-living related Euglenids again encyst [15].

*Viridiplantae*. While higher multicellular plants either form seeds after sexual recombination or haploid spores by meiosis of diploid sporophyte cells for survival and dispersal, encystment is the more common survival strategy for the green algae in this domain. The unicellular green alga *Chlamydomonas* forms thick-walled zygospores when starved of nitrogen. Related multicellular *Chlamydomonales*, such as *Volvox carteri* produce similar zygospores in response to heat shock and drought [16]. For prasinophytes, marine algae covered by scales, cysts were reported for *Pyramimonas gelidicola* [17], while asexual cysts of *Mantoniella squamata*, were recently germinated from 40 year old sediments, and also differentiated from vegetative cells in culture [18].

*Stramenopiles*. This group contains a wide variety of photosynthetic algae, known as phytoplankton, of which many species form cysts that sink to the benthic zone, which acts as a seed bed for repopulation of the water column above (reviewed in [19]). Among them are the diatoms that form asexual cysts, known as hypnospores, that can survive up to nine years [20]. Some species of haptophytes, also marine algae, form cysts, which showed long term survival in sediments [18]. Some chrysophycean algae differentiate into asexual cysts in culture, which are in nature mostly found as empty walls [21], while other species in the group also form sexual cysts known as hypnozygotes. The fungi-like oomycetes, important plant pathogens, differentiate into both asexual motile zoospores, which are used for dispersal, and double-walled sexual oospores, which are used for survival [22].

*Rhizaria*. Within this group of mostly non-photosynthetic planktonic species, the cercozoan *Lecythium* and *Chlamydomphrys* spp. are reported to form cysts [23], while some radiolarian *Acantharea* spp. form sexual cysts [24]. No encystment has been described for the Foraminifera and also many cercozoan and radiolarian taxa do not encyst.

*Alveolates*. Encystment commonly occurs in ciliates in response to starvation, desiccation and other external factors. Encystation involves reduction in cell volume by autophagy and dehydration, metabolic dormancy and encapsulation in a resilient but permeable cell wall. Some species also form zygotic cysts, but this usually occurs under conditions favourable for growth and the cysts rapidly germinate again [25]. The dinoflagellates form both long-lived zygotic cysts (hypnozygotes) and asexual resting cysts, but also thin-walled pellicle cysts that are not long-lived, but there are many variations on this theme within the group [26].

Other members of Alveolata are obligate intracellular parasites. Some such as *Cryptococcus*, *Eimeria*, *Isospora* and *Toxoplasma* form thick-walled zygotic oocysts that are released from their host into the environment, where they survive for some time before infecting a new host [27]. *Toxoplasma gondii* can also form very large cyst-like structures, containing up to 1000 semi-dormant bradyzoites [28], which are at this stage impervious to immune clearance and drug treatment.

*Fungi*. This large domain shows a broad variety of mechanisms for reproduction, survival and dispersal, forming mostly sexual, but also asexual spores. Spore dispersal is facilitated by forceful expulsion from fruiting structures and/or by a covering of hydrophobic proteins which allows spores to become airborne. In the nucleariid amoebas, closest sister group to fungi, cysts have not been reported for solitary species, but *Fonticula alba*, a multicellular member of this group [29] forms both elliptical spores in fruiting structures and round cysts from unaggregated amoebas [30].

*Holozoa*. Metazoa only exist as single cells in the gamete stage and are in this stage typically not metabolically dormant, although hibernation of the whole animal is quite common. However, their unicellular protozoan ancestors are again quite prone to encystment. Among choanoflagellates, thought to be the closest living protists to metazoa, encystment occurs only in freshwater species. In culture, *Desmarella moniliformis* start differentiating into asexual cysts in late log phase, which involves retraction of the collar of villi and flagella, characteristic to the group, into a the flask-shaped cyst wall [31]. Among Filasterea, a class of amoeboid holozoa, *Capsaspora owczarzaki* differentiates into round cysts, but also collects into aggregates, where individual cells become embedded in matrix [32].

*Amoebozoa*. Encystation is particularly widespread amongst the Amoebozoa with many medically relevant species relying on encystation as part of their life cycle. We therefore treat this group in greater detail, starting with the phylogeny of the group and summarizing available data on molecular mechanisms that control encystation in Amoebozoan pathogens.

### Phylogenetic relationships between Amoebozoa

As is the case for protists in general, the classification of species within Amoebozoa was long problematic, due to morphological similarities only poorly reflecting genetic relationships between taxa. The first single gene-based phylogenies segregated species fairly accurately in related groups, but left the deeper connections between these groups unresolved [33, 34]. A recent well-resolved phylogeny inferred from 325 concatenated genes subdivides Amoebozoa with confidence into three lineages: Tubulinea, Evosea and Discosea [12]. The Tubulina contain both the naked and the testate amoebas, the latter surrounded by a body armour fortified with calcium, silicium or other compounds depending on the species. The Evosea containing the amitochondriate Archamoeba, the syncytial Myxogastria, the multicellular Dictyostelia and most protostelid-like amoebas, which form stalked fruiting bodies with one or a few spores from a single cell. The Discosea contain Flabellinia and Centramoebidia as major clades, the latter with the Acanthamoebidae as best known members.

Figure 2 shows a schematic representation of this phylogeny annotated with the presence or absence of other dormant structures in genera for which this information is available. The greater majority of species across all lineages forms dormant cysts, strongly suggesting that this was the long-term survival strategy of the last common ancestor (LCA) of Amoebozoa. However, cysts were only sparsely observed in the order Flabellinia and not at all in Cutosea. Otherwise several genera within orders do not encyst as well as species within otherwise encysting genera.

The ability to aggregate and form multicellular fruiting bodies with spores evolved two times independently – in Evosea in Dictyostelia and in Tubulinea in Copromyxa, while the ability to form spore-bearing structures from a syncytium evolved once in Myxogastria. The paraphyletic protostelids, while mostly members of Evosea, are also scattered across Discosea. Some workers suggest that this indicates that the amoebozoan LCA may have been a protostelid [12], while others consider it more likely that unrelated protostelids evolved independently as stalked cysts [35].

Zygotic cysts have only been observed in Dictyostelia, *Copromyxa* and *Sappinia*. Sexual recombination is an important aspect of the life cycle of *Physarum* and other myxogastrids, but the zygote develops into a syncytium, that can either form a diploid desiccated dormant mass, called a sclerotium, or after meiosis form haploid spores.

However, because sex occurs in at least three orders of Amoebozoa and depends on the complex meiotic machinery that is unlikely to have evolved thrice independently, it is argued to have been present in the LCA to Amoebozoa and either to be cryptic or lost in many species [36, 37]. A similar argument suggests a single origin for encystment in Amoebozoa, despite it not occurring in many species. However, we have limited information to what extent cysts across the phylogeny resemble each other biochemically. In fact, they are known to differ in major wall components like cellulose (*Dictyostelium*, *Acanthamoeba*) or chitin (*Entamoeba*, some Protostelids [38]). Taking also in account the considerable pressures to develop dormancy under e.g. climate change, it remains possible that particular forms of encystment evolved independently within Amoebozoa.

Most Amoebozoa feed on bacteria and unicellular eukaryotes in a wide variety of ecosystems and are harmless to humans. Encystation usually occurs in response to food or water deprivation, other forms of environmental stress or stimuli specific to the habitat. However *Paramoeba spp.* are important pathogens of salmon, lobsters and sea urchins [39, 40] and there are also obligate human parasites and opportunistic human pathogens among the Amoebozoa. The most fearful obligate parasite is *Entamoeba histolytica*, member of the amitochondriate Archamoebae in Tubulinea [12].

### ***Entamoeba histolytica***

Entamoebidae are mostly harmless commensals, which can only survive as feeding amoebas or trophozoites in the colon of animals, where they feed on the bacterial flora. They encyst while passing through the gut into the environment and remain encysted until they reach the colon once more by oral uptake. *E. histolytica* can additionally penetrate the intestinal wall, causing severe bloody diarrhoea, and progress further into the liver and other organs, causing abscesses. *E. histolytica* infection results annually in about 100,000 deaths, second in mortality to malaria [41]. The development of new therapeutics is mostly aimed at killing the trophozoites, but because the cysts are responsible for transmission of the disease, research efforts are also aimed at understanding and preventing encystment. Such studies use *Entamoeba invadens*, a parasite of reptiles, because *E. histolytica* cannot be induced to

encyst *in vitro*. A number of proteins and processes with important roles in encystation have emerged.

Encystation *in vitro* is triggered by glucose depletion and hypo-osmolarity and was also found to be stimulated cholesteryl sulfate and by catecholamines such as adrenaline and noradrenaline [42-44]. Cholesteryl sulfate is a terminal metabolite of sulfate metabolism in *Entamoeba*. Its synthesis is inhibited by chlorate, which also inhibits encystation. While this indicates a potential role for cholesteryl sulfate in encystment, the high concentrations (>0.1 M) of chlorate required for inhibition, exceeding the IC<sub>50</sub> for trophozoite growth lethality may also make cells too sick to encyst.

The catecholamines bypassed bovine serum and cell density requirements for encystment *in vitro* and were specific for  $\beta$ 1-adrenergic receptor agonists. B1-receptor antagonists prevented adrenaline, but not di-butyryl-cAMP induced encystation, suggesting that similar to mammalian  $\beta$ 1-adrenergic receptors, the *Entamoeba* receptors activated an adenylate cyclase [43]. However, the *Entamoeba* genome contains neither adenylate cyclases nor mammalian-type  $\beta$ 1-adrenergic receptors [45, 46], indicating that *Entamoeba* processes the catecholamine signal differently.

Aggregation of cells is a prerequisite for encystation and is mediated by binding of galactose(Gal)-terminated cell surface lectins to receptors on neighbouring cells. It is unclear how this interaction or the other triggers are processed by the cells to execute the encystation programme, which results in expression of enzymes and structural proteins of cyst wall. Chitin fibrils are the main cyst wall component [47]. The fibrils cross-linked and attached to plasma membrane Gal/GalNac lectins by the lectin "Jacob", which contains regularly spaced chitin binding domains. The plasma membrane Gal/GalNac lectins also mediate binding to bacteria and epithelia and contribute to cytolysis and tissue invasion by *Entamoeba*. They also act as receptors for the Gal-terminated lectins that mediate aggregation [48]. Another chitin binding and self-polymerizing lectin "Jessie" makes the cyst wall impermeable, while cysteine proteinase, chitin deacetylase and chitinase contribute to remodelling the cyst wall [49].

Studies with inhibitors for specific heat-shock proteins (HSPs) and cysteine proteases (CPs) indicated that HSP-90 prevents [50] and CPs promote encystation, respectively, although CPs are also required for trophozoite growth [51, 52]. Proteasome inhibitors also have inhibitory effects on encystation [53, 54], but affect trophozoite health in general [55].

Much is still to be learned about the mechanisms that regulate encystation in *Entamoeba*. While the organism can be genetically transformed by plasmid vectors [56], it shows variable polyploidy because cells duplicate their genome without going through cytokinesis and/or nuclear division [57]. The polyploidy of its genome severely hinders gene disruption and forward genetic approaches for gene discovery in encystation. Entamoebidae do have a robust endogenous RNA interference pathway [58] and double stranded RNAi approaches have been successfully used for gene silencing [59]. Additionally, knock-down of protein function by constitutive or inducible expression of antisense RNA, expression of dominant-negative alleles or expression of the 5'flanking region of genes has been successfully applied [60]. Such approaches at the least allow reverse genetic validation of roles for candidate genes suggested by transcriptomic or proteomic studies or of encystation genes discovered in more genetically tractable Amoebozoa.

### ***Acanthamoeba and other free-living amoebozoan pathogens***

Amoebozoa that normally spend their lives in soils or surface waters can occasionally enter humans via oral or nasal routes and cause infections of the central nervous system, or enter the eye and cause vision destroying keratitis. Though relatively rare, the brain infections are

mostly lethal, whereas the eye infections have surged in contact lens wearers that practice poor lens hygiene or used sub-standard lens cleaning solutions [61]. *Acanthamoeba castellanii* and several other *Acanthamoeba* species and *Balamuthia mandrillaris* have been reported to cause granulomatous encephalitis, with a single case caused by *Sappinia pedata* (Flabellinia). *Naegleria fowleri*, a free-living amoeboflagellate, which resides not in Amoebozoa but in Excavata, also invades the brain, causing primary amoebic meningoencephalitis [62], and there is also a report of *Vermamoeba (Hartmannella)* in Amoebozoa, causing this disease [63]. Acanthamoebidae are mostly responsible for the eye infections, affecting 10 per million individuals per year [64], with one reported case for *Dictyostelium polycephalum* [65]. The *Acanthamoeba* trophozoites destroy the corneal epithelium and stroma, and when left untreated result in blindness and/or loss of the eye. Treatment is complicated by encystment of the trophozoites. The metabolically dormant and encapsulated cysts are impervious to immune clearance and antibiotics, requiring prolonged and painful treatment with antiseptics such as chlorhexidine and polyhexamethylene biguanide (PHMB). Trophozoites on the other hand are susceptible to antibiotics like neomycin. Here, drugs aimed to prevent encystment and cause excystment would markedly improve resolution of the infection. Despite this incentive, research into the mechanisms controlling encystation of free-living amoebozoia has not been intensive.

Encystation is in nature induced by starvation, but is also triggered by high osmolarity and by 50 mM MgCl<sub>2</sub> [66, 67]. Cellulose is the major structural component of the inner wall of the double-walled *Acanthamoeba* cyst and enzymes like glycogen phosphorylase, UDP-glucose pyrophosphorylase, and cellulose synthase, which mediate glucose production from glycogen and its subsequent incorporation into cellulose, are highly expressed during encystation [68]. Silencing of glycogen phosphorylase and cellulose synthase expression by RNA interference resulted in formation of immature cysts, that lacked the inner wall [69-71]. Plant cellulose synthase inhibitors, like 2,6-dichlorobenzonitrile and isoxaben, which are widely used as herbicides, also proved effective in preventing formation of the inner cyst wall and cyst maturation, and increased the amoebicidal effect of the antiseptic PHMB [72].

Encystation is also suppressed by the autophagy inhibitors chloroquine and 3-methyladenine [73, 74] and by RNAi mediated silencing of the autophagy proteins Atg8 [75], Atg12 [76] and Atg16 [77]. Atg8 and Atg16 are upregulated in encystation, while Atg12 is already present in trophozoites. Similar to the cellulose synthase inhibitors, the autophagy inhibitors also increased the amoebicidal effect of PHMB [74].

Further evidence for the importance of regulated proteolysis in encystation is provided by observations that gene silencing of a cyst-specific cysteine protease, but also of an endogenous cysteine protease inhibitor (AcStefin) resulted in incomplete encystation [78, 79]. The knock-down of the cysteine protease resulted in incomplete digestion of cellular components in lysosomes, confirming the importance of autophagy for progression of the starving cells through the encystation programme. Gene silencing of a non-lysosomal metalloprotease, M17 leucine aminopeptidase, also reduced encystation as did bestatin, an inhibitor of this class of enzymes [80], indicating that regulated proteolysis during encystation is not restricted to autophagy. Protein methylation also plays a role in encystation, since the protein arginine methyltransferase, PRMT5, which methylates histones, tumour suppressors and many other proteins in humans, is strongly upregulated in encystation, with PRMT5 gene silencing reducing encystment [81].

Most of the regulatory proteins mentioned above were identified from EST sequencing and microarray approaches to detect genes that are overexpressed in cyst compared to trophozoites [82-84]. Many of such genes will be involved in execution of the encystation



programme and not necessarily in the transduction of the external stimuli that regulate this programme. Information on the signalling processes that control encystation is still sparse.

Increased adenylate cyclase activity shortly after induction of encystation suggested a role for cAMP in triggering encystation [85, 86]. In *Vermamoeba* (*Hartmannella*) trophozoites, cAMP levels increased in response to stimulation with  $MgCl_2$  and taurine, two factors that induce encystation, while exposure of trophozoites to cAMP or di-butyryl cAMP induced encystation [87]. Mammalian adenylate cyclase is stimulated by adrenaline via  $\beta$ -adrenergic receptors (BARs). In *Acanthamoeba*, the BAR antagonist propranolol reduced both cell viability and encystation and decreased protease activity. Conversely, the BAR agonist isoprenaline increased extracellular protease activity, but had no effect on cell viability and encystation [88]. While this was concluded to indicate a role for BARs in *Acanthamoeba* physiology, it should be noted that *Acanthamoeba* lacks the 12 transmembrane adenylate cyclases that are the target of BARs [89].

A role for protein kinase C (PKC) is indicated by observations that the PKC inhibitor chelerythrine chloride reduced encystation of *Acanthamoeba* [90] and that 21 out of its 27 PKC genes are upregulated in encystation [82]. Silencing of one of these genes, ACPKC23 resulted in reduced encystation [90]. It is however not known how ACPKC23 activity is regulated and which protein(s) are phosphorylated by this kinase.

### Insights from social amoebas

Dictyostelid social amoebas are well-studied members of Amoebozoa and the model organism *Dictyostelium discoideum* is best known for the fact that its amoebas aggregate when starved to form fruiting bodies with dormant spores and dead stalk cells. It uses secreted cAMP pulses as chemoattractant for aggregation and coordination of post-aggregative cell movement, while secreted cAMP also induces the differentiation of prespore cells. These effects of cAMP are mediated by the G-protein coupled receptor cAR1. cAMP also has a “classic” second messenger role acting on cAMP-dependent protein kinase (PKA), with active PKA being essential for the differentiation of spores and stalk cells and the maintenance of spore dormancy [91, 92]. In this role cAMP is synthesized by the adenylate cyclases ACA, ACG and ACR, but its levels are most critically regulated by the cAMP phosphodiesterase RegA. RegA consists of a mammalian HDc type phosphodiesterase (PDE) domain and a receiver domain that is the target for aspartate phosphorylation/dephosphorylation by histidine-aspartate phosphorelay. This signal transduction pathway, which is common to bacteria, fungi and plants is activated by sensor histidine kinase/phosphatases (SHKPs) [93]. For RegA, phosphorylation of the receiver domain activates the phosphodiesterase activity, decreasing intracellular cAMP levels [94, 95]. In *D. discoideum*, the SHKPs detect signals like the spore-inducing peptide SDF2, ammonia, high osmolarity and the cytokinin, discadenine, that regulate the timely maturation of spores and stalk cells and the maintenance of spore dormancy in the fruiting body [91, 96, 97].

Many Dictyostelids, such as *Polysphondylium pallidum* have retained encystation as an alternative survival strategy to sporulation. Encystation usually occurs when amoebas are submerged or in darkness, two conditions that are unfavourable for aggregation. As in *Acanthamoeba*, high osmolarity (solute stress) is also a trigger for encystation [98]. Evolutionary comparative studies showed that PKA is not only required for sporulation across Dictyostelia, but is also essential for encystation. Knock-out of the PKA catalytic subunit (PkaC) in *P. pallidum*, prevents both starvation- and solute-induced encystation, as does the combined knock-out of the adenylate cyclase ACG and ACR [99]. Conversely, deletion of RegA causes precocious encystation, while the amoebas are still feeding [100]. The PDE

activity of RegA is inactivated by inhibitors of mammalian PDEs, such as dipyridamole and trequinsin. These compounds also inhibit *Acanthamoeba* RegA and cause precocious encystation of *Acanthamoeba*, accompanied by an increase in intracellular cAMP. This suggests that the cAMP-PKA pathway also mediates starvation- and solute-induced encystation in *Acanthamoeba* [100] and possibly other Amoebozoa.

Similar to *Acanthamoeba* (see above), cellulose synthesis is also essential for *P. pallidum* encystment, since disruption of one of its two cellulose synthase genes prevented cyst maturation and rendered cysts inviable [101].

### Insights from comparative genomics

Following the genomes of the Archamoeba *Entamoeba histolytica* and the Eumycetozoan *Dictyostelium discoideum* in 2005 [45, 102], the genomes of the Centramoebia *Acanthamoeba castellanii* [89], the Eumycetozoan *Physarum polycephalum* [103] and the Variosea *Protostelium aurantium* [38] have now been sequenced and annotated. While not representative of all major clades of Amoebozoa, these genomes do represent a large segment of the genetic depth of Amoebozoa and allow us to assess the extent to which genes with known involvement in encystation or signal transduction in general are conserved.

We first analysed to what extent genes controlling *P. pallidum* encystation are also present in other Amoebozoa. Figure 3 shows that the PKA catalytic and regulatory subunits (PkaC and PkaR) are conserved, sometimes with duplicate genes, in *A. castellanii*, the myxogastrid slime mold *P. polycephalum* and the protostelid *P. fungivorum*, but not in *E. histolytica*. The adenylate cyclase ACR is present in *Acanthamoeba* and *Physarum*, but not in *Protostelium* and *Entamoeba*. ACG was not detected outside of Dictyostelia. RegA is again deeply conserved in all Amoebozoan genomes, except *Entamoeba*. Remarkably, RegA is also present in the amoebaflagellate *Naegleria gruberi*, not an Amoebozoan, but an Excavate. *Naegleria* also has PkaC and PkaR genes and several adenylate cyclases and phosphodiesterases (Table 1). There is however no evidence yet for a role of these genes in *Naegleria* encystation, which remains up till now mostly uninvestigated.

*D. discoideum* has 16 SHKPs, which are well conserved throughout the *Dictyostelium* phylogeny. Comparison with other Amoebozoan genomes shows that this number is actually rather modest, since *Acanthamoeba*, *Physarum* and *Protostelium* have respectively 48, 51 and 71 SHKPs. SHKPs are absent from *Entamoeba*, but are also plentiful in *Naegleria*. Adenylate/guanylate cyclase genes, cyclic nucleotide (cNMP) phosphodiesterases and cNMP binding domains, as present in PkaR, are much more abundant in the solitary free-living Amoebozoa and *Naegleria* than in Dictyostelia, but are again absent from *Entamoeba*. Cell surface cAMP receptors were not detected outside Dictyostelia, suggesting that the solitary Amoebozoa cannot detect extracellular cAMP. However, apart from *Entamoeba*, solitary amoebas do have a very well developed machinery for using cAMP in an intracellular second messenger role.

Surprisingly, despite its complex social life cycle, *Dictyostelium* not only has less cAMP signalling proteins, but also less protein kinases, particularly tyrosine kinases, than free-living solitary amoebas. *Entamoeba* has very low signalling complexity with only a single GPCR and single heterotrimeric G-protein, no SHKPs and no cNMP signaling proteins. It does have a similar number of protein kinases as other Amoebozoa. Particularly its lack of sensors such as GPCRs and SHKPs may be a consequence of its parasitic life style, with limited needs for food seeking and environmental sensing. The abundance of sensors in free-living solitary amoeba suggest that interactions with the environment are vast, probably not only restricted to sensing of physical stimuli, prey and predators, but also involving cooperative and

antagonistic interactions within species and with other organisms in their habitat. The additional interactions required for *Dictyostelium* multicellularity may be fairly limited compared to this repertoire.

### **A conserved role for SHKP regulated cAMP signalling in Amoebozoan encystation?**

The abundance of SHKPs, cAMP signalling proteins and the presence of the SHKP regulated cAMP phosphodiesterase RegA in both Amoebozoa and *Naegleria* indicate that histidine phosphorelay acting on cAMP degradation may be a common mechanism for controlling encystation in Amoebozoa and possibly other protists. Most of the adenylate/guanylate cyclases listed in Table 1 have single or multiple transmembrane domains. This suggests that like *Dictyostelium* ACG, which acts as an osmosensor [104], the activity of these cyclases may also be directly regulated by external stimuli.

Unfortunately, the abundance of cAMP signalling proteins is not conducive for identifying roles for cAMP in solitary Amoebozoa by gene knock-out or gene silencing, as there are too many related genes present to provide functional compensation. For similar reasons individual cAMP signalling proteins may be unsuitable as therapeutic targets to prevent encystation. In case of *Acanthamoeba*, only PkaC can thus far be considered as a unique target for encystation inhibitory drugs. However, the proteins phosphorylated by PKA, not yet known for any Amoebozoan, and proteins expressed in response to PKA activation that execute the encystation programme, amongst which may be genes identified from the differential gene expression studies [84], are likely to yield at least some drug targets.

The abundance of tyrosine kinases in Amoebozoan genomes, most of which harbour transmembrane domains, as well as the presence of target SH2 domains for tyrosine phosphorylation, indicates that, like Metazoa, some Amoebozoa use the tyrosine kinases as sensors, possibly also to regulate encystation.

### **Conclusions**

- Organisms throughout all nine eukaryote subdomains differentiate into walled dormant cysts in response to environmental stress.
- Encystment is the only overt differentiation process for most organisms and its universality suggests that the eukaryote last common ancestor could already encyst.
- Despite its universality little is known about the mechanisms that control encystation or excystation in most subdomains.
- Studies have been mostly limited to pathogens in Amoebozoa, and most progress has been made with differential display of encystation specific genes and knock-down by RNA interference of such genes.
- The molecular mechanisms controlling sporulation in fruiting bodies of the social amoeba *Dictyostelium discoideum*, a genetic model system, have been largely elucidated. Secreted sporulation-inducing signals act on sensor histidine kinases/phosphatases that regulate intracellular cAMP levels by controlling the activity of the cAMP phosphodiesterase RegA. Activation of PKA by cAMP causes spore encapsulation and prevents precocious spore germination.
- Comparative studies showed that this pathway also mediates stress-induced encystation of individual amoebas, that occurs in some Dictyostelia. The pathway components ACR, PKA and RegA are deeply conserved in Amoebozoa and sensor histidine kinases/phosphatases are plentiful in their genomes. RegA also controls encystation of the

distantly related *Acanthamoeba*, indicating that stress-induced cAMP elevation and PKA activation widely controls Amoebozoan encystation.

- It is however well possible that there are other signalling transduction pathways acting in parallel to PKA and that these pathways play more prevalent roles in e.g. *Entamoeba* and protists outside Amoebozoa.
- Broader development of molecular genetic tools for clade-representative species, which allow gene discovery and validation by forward and reverse genetics, is of primary importance for understanding this most prevalent eukaryote differentiation pathway.

## AUTHOR STATEMENTS

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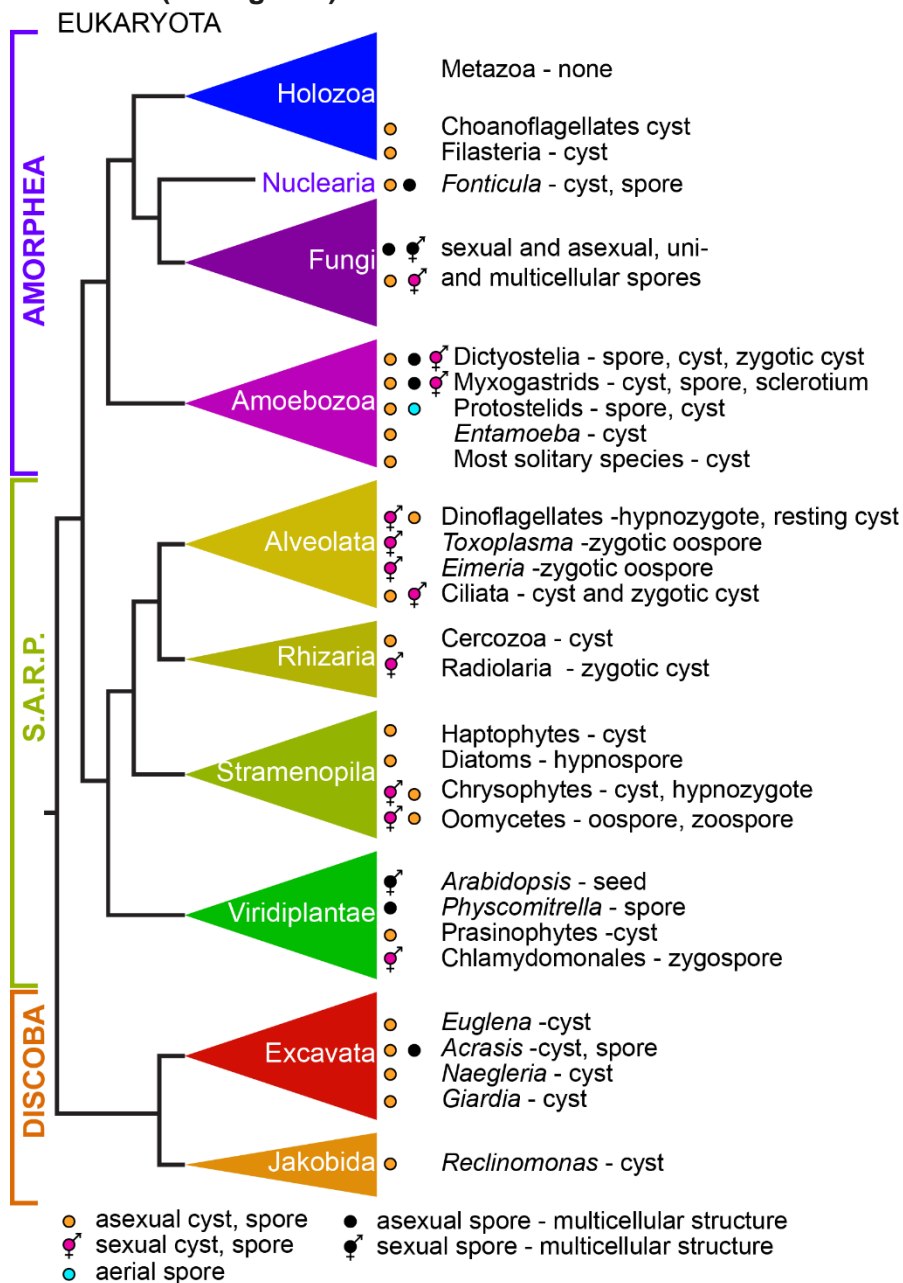
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**LEGENDS (and figures)**

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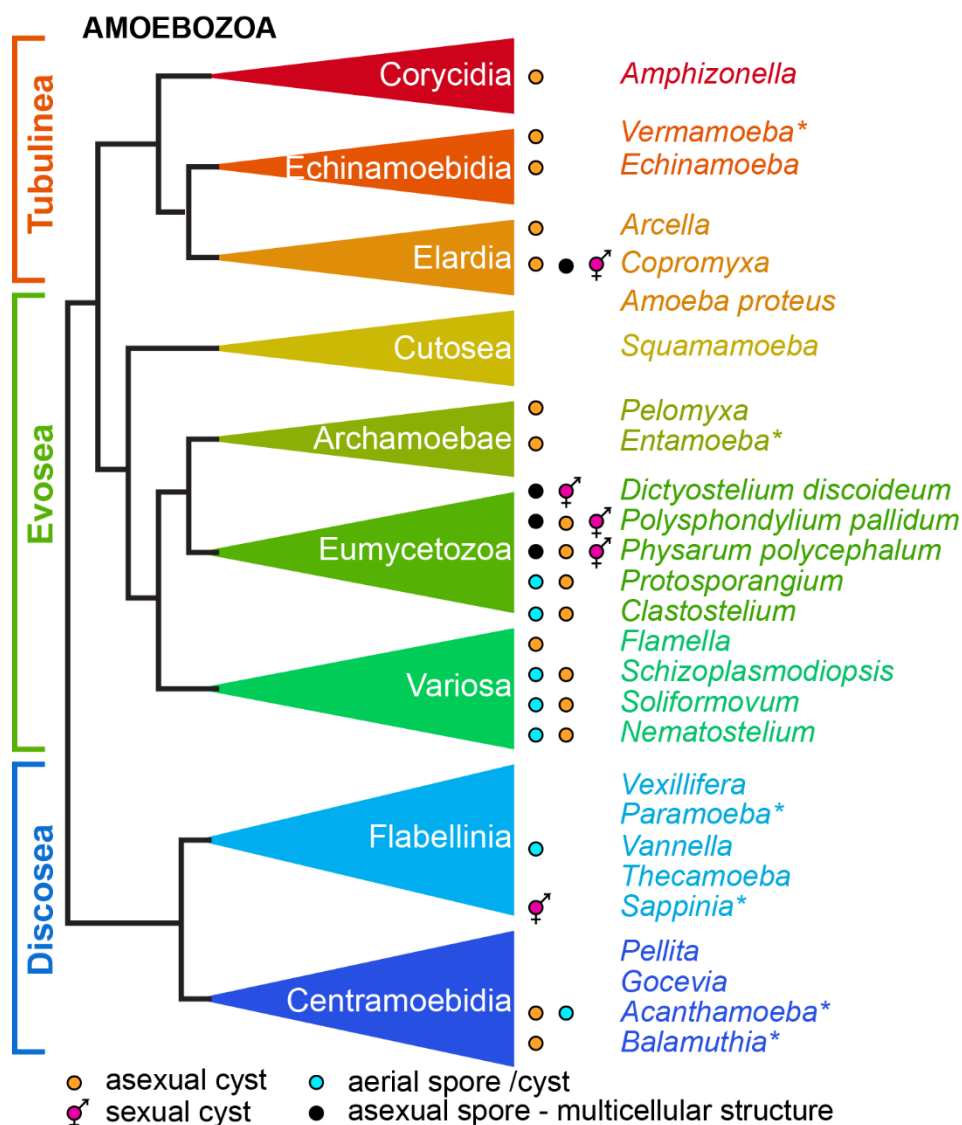
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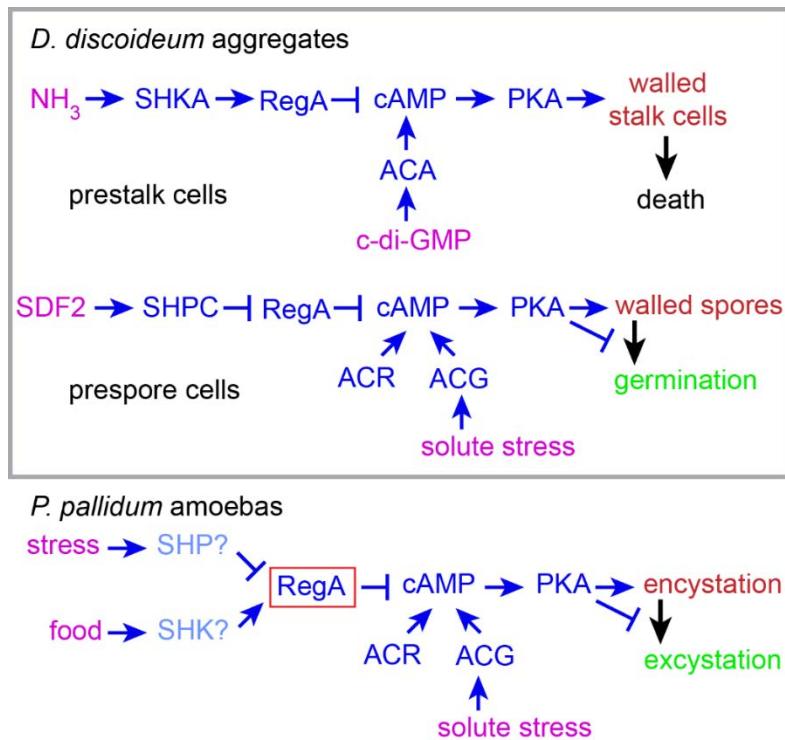
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**Figure 1. Dormant cells across the eukaryote phylogeny.** The eukaryote phylogeny was schematically reproduced from a recent 37 gene phylogeny [11], with Rhizaria added as sister clade to Alveolata [105]. Genera (*italics*) or higher order groups of species with documented sexual or asexual dormant cysts or spores are indicated. Note that often not all species within the genus or group have a dormant stage.

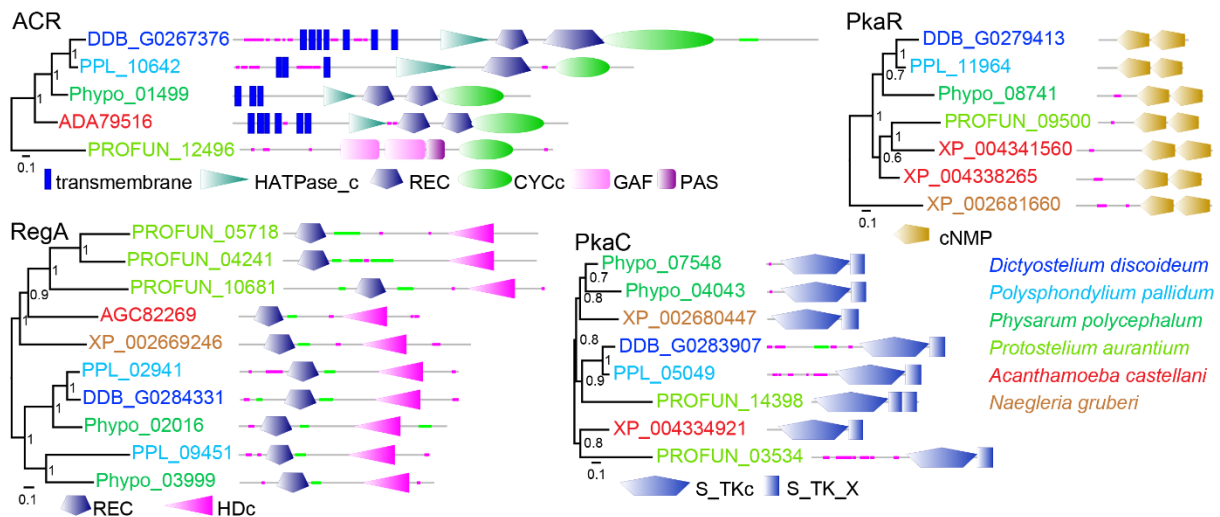


**Figure 2. Encystment and sporulation in Amoebozoa.** The occurrence asexual and sexual cysts and aerially born cyst or spores was mapped onto the schematically reproduced phylogeny of all Amoebozoa as determined from 325 genes [12]. Data on the occurrence of dormant stages in different genera of Amoebozoa were retrieved from Microworld (<https://www.arcella.nl>. [106]) and the Eumycetozoon project (<http://slimemold.uark.edu/index.htm>, <http://www.discoverlife.org>). \*(opportunistic) pathogens.



**Figure 3. A cAMP signalling pathway controls cell encapsulation.**

*D. discoideum* spore and stalk cell maturation is controlled by secreted stimuli (in pink), with c-di-GMP inducing and ammonia inhibiting stalk maturation, SDF2 inducing spore maturation and high osmolarity inhibiting spore germination. These signals act on either cAMP synthesis by ACR and ACG or on cAMP hydrolysis by RegA via sensor histidine kinases/phosphatases (SHKs/SHPs), which respectively activate/inhibit RegA activity [107]. The same pathway, operates to activate encystation and prevent excystation in response to stress in *P. pallidum*, a dictyostelid which has retained the ancestral encystation pathway. RegA also negatively regulates encystation in the distantly related *A. castellanii*. Involvement of pathway components in dark blue was shown by gene knock-out. Those in light blue are inferred from their abundance in Amoebozoan genomes (see Table1).



**Figure 4. Conservation of *Polysphondylium* encystation genes across Amoebozoa.**

Best bidirectional BLASTp hits for *P. pallidum* cAMP signalling genes that control encystation were identified from the indicated genomes. Phylogenetic trees were inferred from aligned sequences using MrBayes and annotated with the functional domain architecture of the proteins.

**Table 1. Cell signalling proteins in Amoebozoa and *Naegleria***

Category	<i>Acanthamoeba</i> <i>castellani</i>	<i>Dictyostelium</i> <i>discoideum</i>	<i>Entamoeba</i> <i>histolytica</i>	<i>Physarum</i> <i>polycephalum</i>	<i>Protostelium</i> <i>aurantium</i>	<i>Naegleria</i> <i>gruberi</i>
Histidine kinases/phosphatases	48	16	0	51	71	27
G-protein coupled receptors	35	55	1	146	17	121
Heterotrimeric G-proteins						
alpha	6	12	1	26	9	39
beta	n.d.	1	1	1	1	1
gamma	n.d.	1	2	1	1	n.d.
Cyclic nucleotide signaling						
adenylate/guanylate cyclases	67	5	0	64	52	108
cNMP binding domains	7	5	0	28	27	7
cNMP phosphodiesterases	10	7	1	11	16	7
Protein kinases						
ser/thr and tyr kinases	377	295	307	447	827	265
tyrosine kinases	22	0	55	4	167	89
SH2 domain proteins	48	15	5	18	85	n.d.

Enumeration of different categories of sensors and signal transduction proteins for five Amoebozoan genomes and for the Excavate *Naegleria gruberi*. Data for *Acanthamoeba*, *Dictyostelium*, *Physarum*, *Protostelium* and *Naegleria* were retrieved from [89], [108], [103], [38] and [109] and for *Entamoeba* protein kinases, G-protein coupled receptors, G-proteins and other signalling proteins from [110], [111], [112] and [45], respectively.